LETTER TO JMG

Functional and genetic studies demonstrate that mutation in the COX15 gene can cause Leigh syndrome

C E Oquendo, H Antonicka, E A Shoubridge, W Reardon, G K Brown

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Leigh syndrome is a subacute necrotising encephalomyelopathy characterised by delayed onset of symptoms, hypotonia, feeding difficulties, failure to thrive, motor regression, and brain stem signs. The main laboratory findings are raised lactate in the blood and cerebrospinal fluid, but the diagnosis is only confirmed by the presence of bilateral symmetrical lesions in the basal ganglia, thalamus, brain stem, and cerebellum. Leigh syndrome can result from a number of different defects in mitochondrial energy metabolism, most commonly deficiencies of cytochrome oxidase (COX), pyruvate dehydrogenase, NADH-ubiquinone oxidoreductase (Complex I) and ATP synthase.[1] In patients with Leigh syndrome and cytochrome oxidase deficiency, the underlying genetic defect is usually a mutation in the SURF1 gene, which maps to chromosome 9q34 and encodes a cytochrome oxidase assembly factor.[2 3] In a small number of cases, Leigh syndrome and cytochrome oxidase deficiency have been found in patients with mutations in mitochondrial DNA (mtDNA)[4 5] and in one patient with mutations in the COX10 gene.[6]

Cytochrome oxidase is the terminal complex of the electron transport chain. It transfers electrons from cytochrome *c* to molecular oxygen and contributes to the proton motive force used in the generation of ATP. The mammalian complex is composed of thirteen subunits, three encoded in mtDNA and ten in nuclear DNA.[7] Some of the nuclear subunits have different isoforms, which are coded by multigene families and are expressed in different tissues and at different stages of development.[8–14] The cytochrome oxidase complex contains four prosthetic groups, haeme *a*, haeme a_3, Cu_A and Cu_B, which are essential for the redox reaction. Therefore, generation of a fully functional complex in the inner mitochondrial membrane requires: (*a*) transcription and translation of 13 proteins in two different compartments of the cell, (*b*) correct assembly in the inner mitochondrial membrane with prosthetic groups, and (*c*) regulation of activity according to specific tissue and developmental requirements.

As a consequence of this complexity, it is not surprising that cytochrome oxidase deficiency is genetically and phenotypically highly heterogeneous. At present, a specific genetic diagnosis is made in only ≈50% of patients and the majority of these belong to the SURF1 deficiency group.[15] Mutations in other nuclear genes whose products are necessary for cytochrome oxidase biogenesis, such as SCO1,[16 17] SCO2,[18] COX10,[17] COX15,[19] LRPPRC,[20] TK-2,[21] DGK-2,[22] and thymidine phosphorylase,[23] appear to be much less common. They have been associated with lactic acidosis and non-specific encephalopathy, in some cases co-existing with dysfunctions of other highly metabolic organs, such as heart, liver, kidney, and muscle.

We describe a patient who presented with Leigh syndrome as a result of cytochrome oxidase deficiency, in whom the underlying cause is a mutation in the COX15 gene, which codes for an enzyme involved in haeme *a* biosynthesis. This is only the second patient described with a mutation at this locus and the clinical presentation differs significantly from the previously reported case, suggesting that this condition may be more variable in presentation than other defects of cytochrome oxidase assembly.

Key points

- A patient with typical clinical and neuroradiological features of Leigh syndrome and cytochrome oxidase deficiency was found to have a mutation in the COX15 gene.
- This was initially identified by functional complementation of the enzyme defect in the patient's cells when COX15.1 protein was expressed from a retroviral vector.
- Mutation analysis of the COX15 gene revealed that the patient was homozygous for a missense mutation, cC700T (R217W). Both parents were demonstrated to be heterozygous for the mutation.
- Cytochrome oxidase deficiency, presenting as Leigh syndrome or as a non-specific encephalopathy, is genetically heterogeneous. Initial screening for functional complementation following expression of candidate gene products, as in this case, may be an efficient method for establishing a specific genetic diagnosis.

MATERIALS AND METHODS

Patient cell cultures and derived cell lines

Primary fibroblast cultures were established from skin biopsies and cultured in Dulbecco's Modified Eagle Medium—Nutrient Mixture F-12 Ham (DMEM/F12) supplemented with 10% fetal calf serum and antibiotics. Cultures were transduced with a retroviral vector containing the E6/E7 genes from the type-16 human papilloma virus.[24] Transduced cells were cultured in high glucose Dulbecco's Modified Eagle's medium supplemented with 10% fetal calf serum.

Biochemical analysis

Primary fibroblasts were assayed for cytochrome oxidase activity spectrophotometrically by following the oxidation of reduced cytochrome *c* as described previously.[25] Cytochrome oxidase activity was also demonstrated by cytochemical staining based on mitochondrial localisation of polymerised diaminobenzidine following oxidation by cytochrome *c*.[26] For complementation analysis with retroviral vectors, cellular extracts were prepared and cytochrome oxidase activity determined as described previously.[27] In both protocols,

Abbreviations: COX, cytochrome oxidase; CS, citrate synthase

cytochrome oxidase activity was related to protein concentration and citrate synthase (CS) activity.[28]

Genetic analysis

The coding regions of the SURF1, SCO1, SCO2, and COX10 genes were sequenced following amplification of cDNA or genomic DNA. There are two transcripts from the COX15 gene, COX15.1 and COX15.2, which are generated by alternative splicing.[29] Primers specific for the COX15.1 variant were used to analyse the coding region of this gene in the patient. Messenger RNA was extracted from cultured fibroblasts using a Roche High Pure RNA Isolation Kit and cDNA made with an Omniscript™ RT Kit (Qiagen). Overlapping segments of the relevant coding regions were amplified by PCR, assessed by running an aliquot on 1% agarose gels and purified using a QIAquick PCR Purification Kit (Qiagen). Primers, product sizes, and annealing temperatures are presented in table 1. The products were sequenced using ABI™ Big Dye Terminators (Applied Biosystems). Genomic DNA was prepared from fibroblasts using a Nucleon BACC2 Kit (Nucleon Biosciences). PCR primers were designed to span the relevant exon of the COX15 gene to confirm the mutation in genomic DNA and the PCR products were assessed on agarose gels, purified, and sequenced as before. In addition, the COX15 mutation was demonstrated by StyI digestion of the exon 5 PCR product.

Retroviral transfection

cDNAs corresponding to a number of human cytochrome oxidase assembly genes (COX11, COX15.1, COX17, OXA1, and PET191) were amplified by PCR and cloned into a retroviral expression vector pLXSH using the Gateway cloning system (Invitrogen). Retroviral constructs were used to transfect the GP+E86 ecotropic packaging cell line,[30] and the virus produced was used to infect the amphotropic packaging cell line PA317[31] to create stable virus producing lines. Fibroblasts were infected by exposure to medium containing the virus in the presence of polybrene.[32] Cultures were subsequently selected in 100 U/ml of hygromycin and surviving cells assayed for cytochrome oxidase activity.

RESULTS

Clinical presentation

The patient—a boy—was born at 41 weeks with a birth weight of 3.95 kg. He was initially healthy, but from the age of seven months he was noticed to be hypotonic. Subsequently, he developed spasticity of the lower limbs, feeding difficulties, and horizontal nystagmus. At 15 months of age he showed motor regression, progressive microcephaly, and retinopathy. He was aware of his surroundings but unable to interact with others. At 2 years and 10 months, bloody bowel motions and loss of weight were reported, and these persisted until his death from pneumonia at 3 years, 11 months. There is no apparent history of consanguinity in the family.

Urea, electrolytes, ammonia, lysosomal enzymes, organic acids, very long chain fatty acids, and amino acid chromatography were all normal. Blood lactate was 5.2 mmol/l (normal range: 0.63–2.44) and cerebrospinal fluid lactate was 6.2 mmol/l (normal range: 0.9–2.8). Magnetic resonance imaging of the brain undertaken at 1 year of age (fig 1) showed bilateral lesions in the basal ganglia, dorsal midbrain, cerebral peduncles and periaqueductal region compatible with a diagnosis of Leigh syndrome. Gastrointestinal and muscle biopsies were normal. Cytochrome oxidase activity was below the level of detection in the spectrophotometric assay (<1 nmol/mg protein/min, normal control range, 30–90) and in the cytochemical staining (fig 2).

Mutation analysis and retroviral transfection

In the initial genetic investigation of this patient, mutations were sought in genes known to be involved in defects of cytochrome oxidase biogenesis, SURF1, SCO1 and SCO2, and COX10. The sequence of the SURF1 coding region was normal although there were three repeats of the sequence TGCGGGG in intron 1 of the SURF1 gene. This is a polymorphic variant as either three or four repeats are commonly found in normal controls. SCO1 and SCO2 analysis showed only the normal sequence without any polymorphism. Sequencing of COX10 showed three polymorphisms in an otherwise normal sequence; G1223A (rs1050214), C1384T (rs1050215), and G1111A (which we have identified previously in several normal controls).

To assess the possibility of mutations in other cytochrome oxidase assembly factors, the E6/E7 patient line was transfected with retroviral vectors containing the coding regions of COX11, COX15.1, COX17, OXA1, and PET191. Overexpression of these genes showed that only COX15.1 could rescue the biochemical defect. COX/CS activity, expressed as a percentage of control, showed that the activity of the E6/E7 patient line was increased from 30% (standard deviation, 4%) (n = 3), to nearly normal levels after transfection: 95% (8%) (n = 4).

The sequence of the COX15.1 cDNA from the COX15 gene revealed an apparently homozygous single base substitution, cC700T. This mutation would change an arginine to tryptophan (R217W) in exon 5. This arginine residue is conserved across a wide variety of species, including mammals, insects, plants, and fungi. The presence of the mutation was confirmed by sequencing of exon 5, from genomic DNA, which showed similar results (fig 3). The mutation introduces a restriction site for the enzyme StyI and the presence of the mutation was further verified by digesting the exon 5 genomic fragment with this enzyme. The patient DNA was fully digested into two fragments, of 402 bp and 196 bp (fig 4). Homozygosity was confirmed by PCR amplification, sequencing and restriction enzyme digestion of exon 5 from both parents, who were found to be heterozygous for the same mutation (figs 3 and 4).

Table 1 Conditions for PCR amplification of genes involved in cytochrome oxidase biogenesis

Gene	Fragment	Reaction	Forward primer	Reverse primer	Annealing	Size
SURF 1 (9 exons)	Exons 1–2	gDNA-PCR	5′ tcgtgacctgtcaggatgc	5′ cagacagcaggtggctctg	56°C	736 bp
	Exons 3–9	RT-PCR	5′ aggacgtcctcagggtc	5′ catgatccagcataaaggca	61°C	897 bp
COX10 (7 exons)	Exons 1–5	RT-PCR	5′ gtcccgtgaggagagaggac	5′ ccaaggtcagaatggcaact	62°C	817 bp
	Exons 6–7	RT-PCR	5′ ttttgaggtgccatttgactc	5′ aacccagcaatttctcttcttg	60°C	797 bp
SCO1 (6 exons)	Exon 1	gDNA-PCR	5′ gatggacagagcgactcctt	5′ aactgggactacccgcaag	61°C	552 bp
	Exons 2–6	RT-PCR	5′ agtacccggacgagttatgc	5′ tgcgagacagtttccttcct	60°C	948 bp
SCO2 (2 exons)	Exons 1–2	RT-PCR	5′ gcttcctctcgtgcttggt	5′ taaacgcagcccgtttaatg	61°C	901 bp
COX15.1 (9 exons)	Exons 1–6	RT-PCR	5′ ttgtggaagaggtggctgtt	5′ agacctgctgttccatgagc	58°C	842 bp
	Exons 5–9	RT-PCR	5′ gacgtgttcttgccctctgt	5′ ttgaccatttggaaaccactt		808 bp
	Exon 5	gDNA-PCR	5′ caagatcccgccactg	5′ gtccccatttaacgaacaat	60°C	598 bp

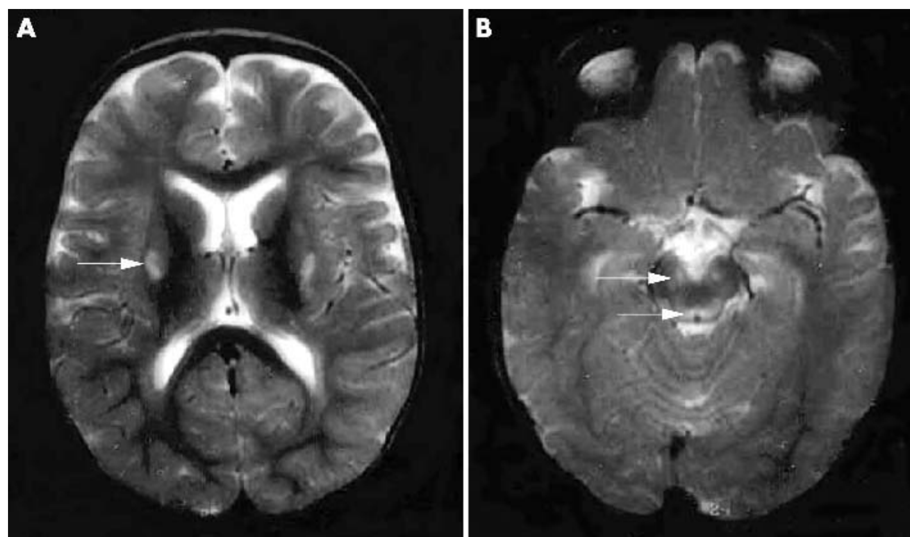

Figure 1 Magnetic resonance imaging of the brain of the patient at age 1 year showed bilateral lesions (indicated by arrows) in the putamina (A) and cerebral peduncles and periaqueductal region (B).

DISCUSSION

It has been established by biochemical studies in *S. cerevisiae* that COX15 protein is required for the biosynthesis of the haeme *a* component of the cytochrome oxidase complex. COX15 is part of a three component mono-oxygenase (ferredoxin, ferredoxin reductase, and COX15) that catalyses the hydroxylation of the methyl group at position eight of the protohaeme molecule.[33] This reaction is followed by oxidation of the subsequent alcohol into an aldehyde, thought to be carried out by an as yet unknown enzyme. In contrast to the yeast, where there is a single COX15 transcript, the human orthologue has two splice variants, COX15.1 and COX15.2, which differ in the C-terminal domain of the protein and the 3′- UTR of the transcript. The significance of these two splice variants in human beings is at present unknown.[29]

Human cytochrome oxidase deficiency due to mutation in the COX15 gene has recently been identified in a patient with fatal infantile hypertrophic cardiomyopathy and seizures.[19] The patient was found to be a compound heterozygote with a splicing mutation in intron 3 (C447 -3G) on one allele and the same missense mutation as found in the present patient on the other. In this second patient, who was homozygous for a R217W mutation in the COX15 gene, there was a more specific effect on brain function, as the presentation and neuroradiology were characteristic of Leigh syndrome. The identification of another genetic cause of this syndrome increases still further the genetic heterogeneity of this condition in particular, and of cytochrome oxidase deficiency in general. The frequency of COX15 mutation is at present unknown. In 25 cytochrome oxidase deficient patients studied to date, functional complementation by a COX15.1 retroviral vector has resulted in identification of one other case apart from the one reported previously[19] and the present patient.

Genetic heterogeneity in Leigh syndrome is well established. It is known that enzymatic defects at a number of different stages of substrate oxidation and oxidative phosphorylation can result in a similar pattern of neuropathological lesions, categorised as ''Leigh or Leigh-like''. However, because the distribution of such lesions can vary substantially from one case to another, attempts have been made to correlate specific neuroradiological patterns with the different genetic defects. Although there is broad overlap, brain stem and subthalamic lesions have been commonly found in patients with SURF1 mutations,[34] while basal ganglia lesions have been reported to be more common in non-SURF1

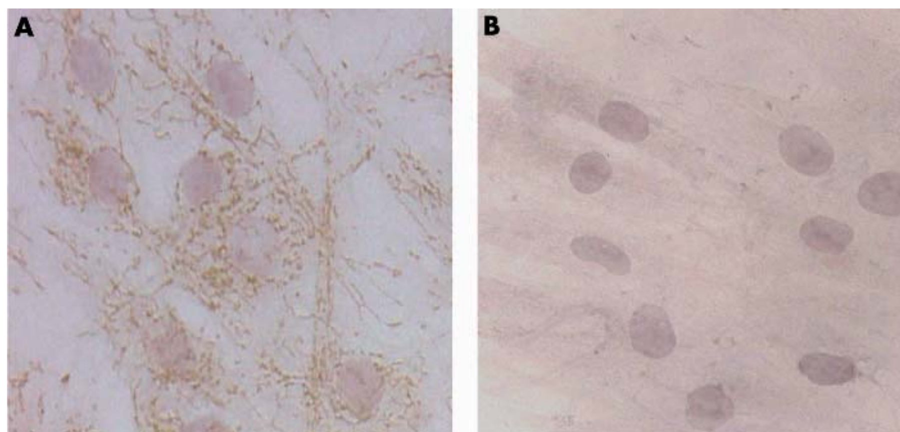

Figure 2 Cytochrome oxidase enzyme cytochemical staining of primary fibroblasts. In the normal control fibroblasts (A), cytochrome oxidase activity is demonstrated as a deposit of specific brown reaction product in a mitochondrial distribution. In the patient's fibroblasts (B), there is no detectable specific reaction product. The cell nuclei are counter stained with haematoxylin.

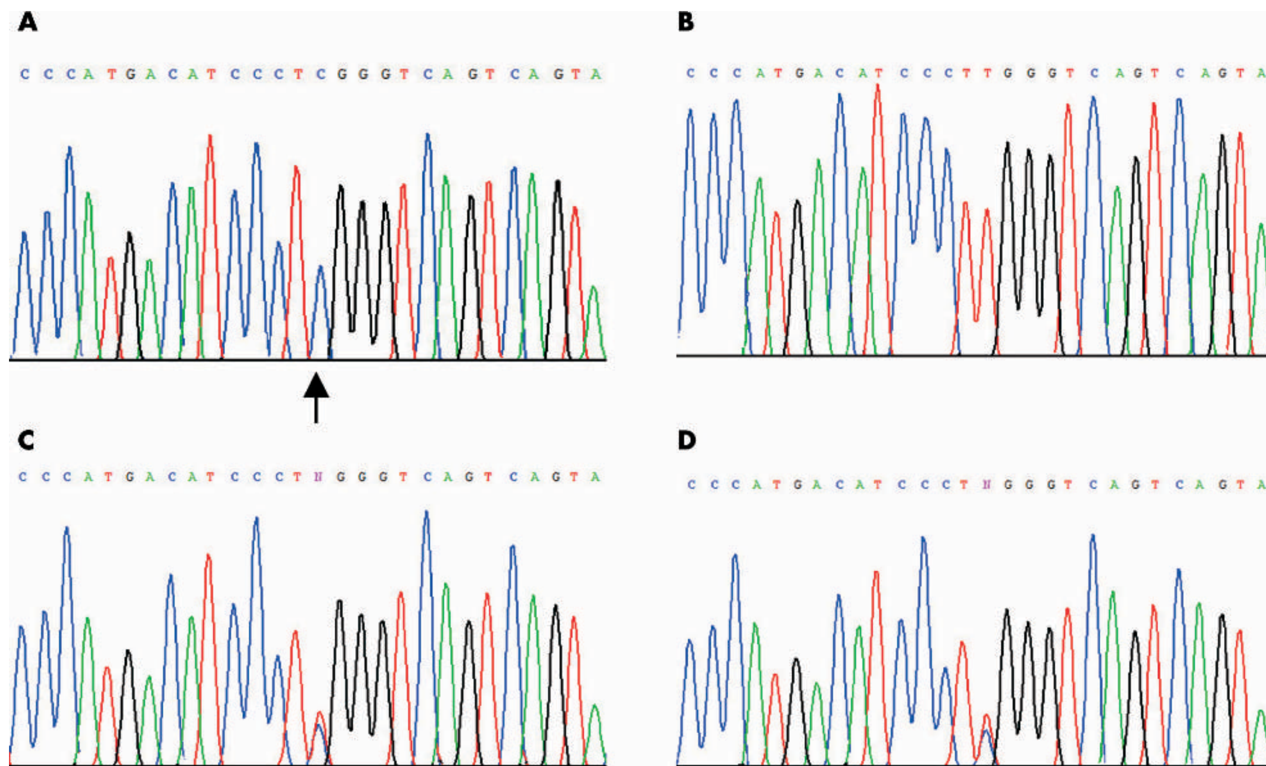

Figure 3 COX15.1 sequencing in genomic DNA. Exon 5 of the COX15.1 gene in a normal control (A), the patient (B), and his parents (C and D). The site of the mutation is shown by the arrow in the normal control sequence. The patient is apparently homozygous for a single base substitution of T for C. The same substitution is present, in heterozygous form, in both parents.

cytochrome oxidase deficient patients and patients with defects in Complex I or Complex III, combined respiratory chain defects, pyruvate dehydrogenase deficiency, or mtDNA mutations.[35] In the patient described here, neuroradiological lesions were found in both the basal ganglia and brain stem, indicating that it is not possible to predict a likely genetic cause from the pattern of neuropathology.

It is also important to note that in many instances, these lesions in the brain are part of a wider condition in which function of other systems, such as the haematopoietic system,[6] cardiovascular system,[36] extrapyramidal tracts,[37] peripheral nerves,[38] or the liver,[39] is also compromised. In this patient with Leigh syndrome due to a mutation in COX15, there were also symptoms and signs of gastrointestinal tract dysfunction, although the pathological basis for this was not determined. This is a novel association of unknown significance, however gastrointestinal dysfunction is a feature of some mitochondrial diseases, particularly MNGIE syndrome,[40] so it is possible that there is a causal connection.

Initial descriptions of patients with mutations in genes for cytochrome oxidase assembly factors suggested the possibility that different gene defects might be associated with specific patterns of tissue involvement, even though there is usually a systemic biochemical defect.[16–19] Subsequent patient reports, including the present one, indicate that there may be considerable clinical and biochemical heterogeneity among patients with mutations in the same gene.[6] As the number of cases of individual genetic defects is at present quite small, it will require identification of many more cases before it is clear whether any are associated with a specific phenotype. In patients with typical Leigh syndrome and cytochrome oxidase deficiency, analysis of the SURF1 gene is indicated in the first instance. However, in other patients it may prove that a general screening method, such as the retroviral

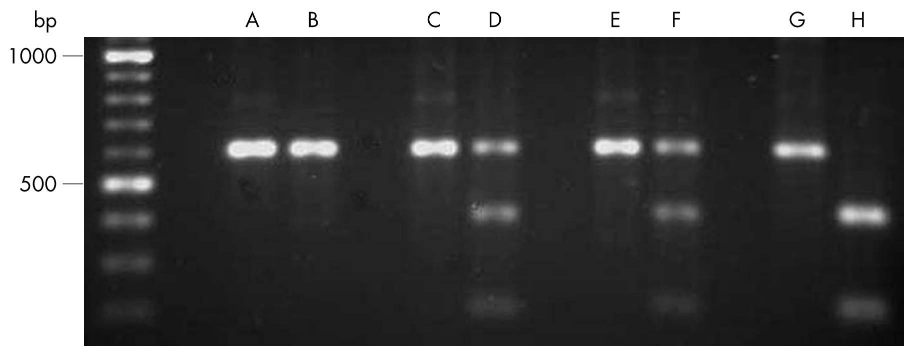

Figure 4 StyI restriction digests of COX15.1 exon 5. The COX15.1 exon 5 PCR product was analysed for the presence of the StyI site which is generated by the mutation. Paired samples are shown of a normal control (A, B), the patient's father (C, D), patient's mother (E, F) and the patient (G, H). In each case the undigested PCR product is on the left and the StyI digest to the right.

expression system used in this case, will be particularly useful as the genetic defect may not be predictable from the clinical and biochemical presentation.

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Authors' affiliations

C E Oquendo, G K Brown, Genetics Unit, Department of Biochemistry, University of Oxford, UK

H Antonicka, E A Shoubridge, Department of Human Genetics, McGill University, Montreal, Canada

W Reardon, Our Lady's Hospital for Sick Children, Dublin, Ireland

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Correspondence to: Dr G K Brown, Genetics Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, UK; garry.brown@bioch.ox.ac.uk

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